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Adenylate Cyclase Toxin Is Critical for Colonization and Pertussis Toxin Is Critical for Lethal Infection by *Bordetella pertussis* in Infant Mice

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Proliferation of *Bordetella pertussis* in the lungs of infant mice challenged by the intranasal route was examined. The bacteria rapidly proliferated in the lungs of mice challenged with a sublethal dose of a wild-type strain (BP338) or a filamentous hemagglutinin mutant (BPM409) from 500 at day 0 to 10^7 at day 15. The infection cleared in about 40 days. Pertussis toxin-deficient mutant BP357 gave a similar profile; however, the number of bacteria recovered was slightly reduced, suggesting that pertussis toxin is not essential for bacterial growth in the lungs. In contrast, adenylate cyclase toxin mutant BP348 was rapidly cleared from the lungs, with no viable bacteria remaining 10 days postchallenge, suggesting that the adenylate cyclase toxin is a colonization factor required for the bacteria to initiate infection.

Previous studies have shown that *Bordetella pertussis* mutants that fail to produce only the filamentous hemagglutinin (FHA) are unaltered in virulence, while mutants that fail to produce the adenylate cyclase toxin, or pertussis toxin, are severely deficient in the ability to cause lethal infection in infant mice challenged intranasally (7). To determine how the disease process was altered in the mutants, 6-day-old BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine) were infected intranasally as described previously (7). The mice were killed by methoxyflurane inhalation (5 days postinfection) or cervical dislocation (all other times), and the lungs were removed. Colony counts were determined by suspending the lungs in 5 ml of Stainer-Scholte broth (6), disrupting them for 60 s in a model 80 Stomacher laboratory blender (Seward Medical, London, England), and plating various dilutions on Bordet-Gengou agar (7).

Challenge with a sublethal dose of wild-type strain BP338 (approximately 500 bacteria) resulted in rapid proliferation of the bacteria to a peak of about 10^7 at day 15, which gradually resolved by day 40 (Fig. 1). In contrast, no bacteria were detected in the lungs 48 h postchallenge with 10^7 cells of BP347, an avirulent-phase mutant which is unable to express any of the virulence-regulated genes (data not shown). This demonstrates that failure to express any of the virulence-regulated genes results in rapid elimination of the bacteria from the respiratory tract.

To understand the contributions of individual virulence factors in the disease process, experiments were performed with mutants deficient in one of the virulence factors. The insertions in the adenylate cyclase and FHA genes map to the structural gene, and the mutants totally lack antigenic production of the respective factors. The pertussis toxin insertion maps to the end of the structural gene and is deficient in production of the toxin but not negative (8, 9). The greater dose required to cause a lethal infection, 1,000-fold that of the wild type, suggests that the deficiency in the pertussis toxin mutant is severe, but further studies with a deletion mutant would be useful to confirm these results.

Mutants unable to produce FHA are as virulent as the wild-type strain (7). Recovery rates similar to those obtained with the wild-type strain were seen when the mice were challenged with BPM409, an FHA mutant (Fig. 1). Only low challenge doses were used for the wild-type strain and FHA mutant, since higher doses would have caused a lethal infection.

Pertussis toxin mutant BP357 is severely impaired in the ability to cause a lethal infection (7). In low-dose challenges (Fig. 1), during the first 10 days of the infection it did not appear to be different from the wild-type strain. After this time, no further growth was detected. The number of bacteria in the lungs peaked at 10^6 as opposed to 10^7 for the wild-type strain, and the infection took 25 to 30 days instead of 40 days to clear. When a higher challenge dose (5×10^4) was used, greater numbers of bacteria were recovered from the lungs of the mice and the infection persisted as long as that caused by the wild type (Fig. 2). These data suggest that pertussis toxin is not required for initial replication of the bacteria but appears to play a critical role in the later stages of infection.

In contrast, when the mice were challenged with a low dose of adenylate cyclase mutant BP348, which is also severely impaired in the ability to cause a lethal infection (7), very little bacterial replication was seen and the mice were able to clear the bacteria from their lungs in 10 days (Fig. 1). A different result was obtained when a high challenge dose of BP348 was used (Fig. 2). Bacterial replication was seen, and instead of rapid clearing of the bacteria from the lungs, a persistent infection was set up that lasted even longer than the 40 days seen with the wild-type strain.

All of the isolates recovered from the lungs of the mice were tested for fimbrial production (Table 1). Monoclonal antibodies were used to type the strains as described previously (9). No isolates were found to express fimbrial agglutinogen 3/6 (21.5-kilodalton subunit size) when monoclonal antibody BPC10 was used (4). Fimbrial agglutinogen 2 (22-kilodalton subunit size) was detected with monoclonal antibody BPF2 (4). As observed in previous studies, expres-

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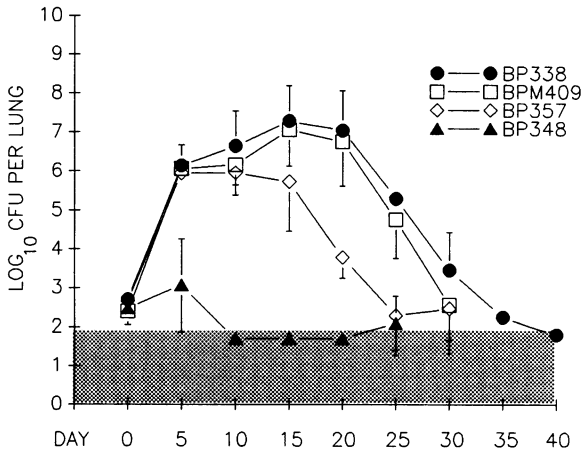

FIG. 1. Recovery of bacteria from the lungs of mice infected with a low-dose challenge (approximately 500 bacteria). The strains used in this study, BP338 (wild type), BP347 (avirulent phase) (data not shown), BP348 (adenylate cyclase toxin deficient), BP357 (pertussis toxin deficient), and BPM409 (FHA deficient), were described in previous studies (8, 9). The geometric mean and standard deviation of at least three mice per data point were plotted, except for days 25 and 35, when BP338 was used and the averages of two experiments were plotted and the standard deviations were not calculated. The shading denotes the area below the limit of detection (50 bacteria per lung).

sion of serotype 2 fimbriae could vary during the course of the infection, suggesting that this factor does not play an essential role in the disease process.

The virulence of a bacterial mutant unable to synthesize a single virulence factor is somewhat analogous to what happens when an animal is immunized with a single protein and then infected with a wild-type strain. This analogy works well when neutralization of an activity is required for protection but breaks down when opsonizing antibodies are induced, since these antigens could be irrelevant to the disease process but important for protection. This study starts to address the roles of certain factors in the disease process and as protective antigens.

Consistent with our previous studies, production of FHA did not appear to be essential for lethal infection of infant

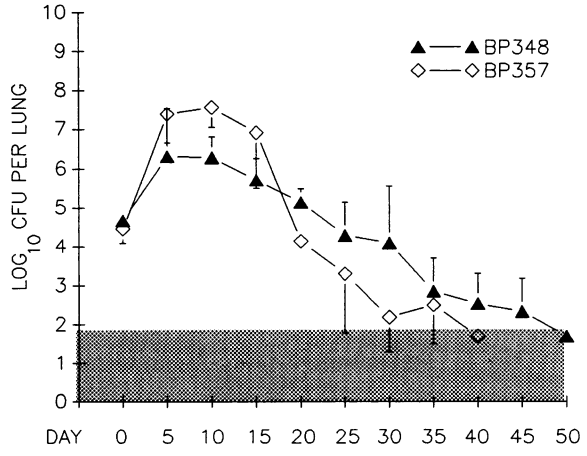

FIG. 2. Recovery of bacteria from the lungs of mice infected with a high-dose challenge (approximately 5×10^4 bacteria). These studies were performed as described in the legend to Fig. 1.

TABLE 1. Fimbrial serotypes of isolates recovered from mouse lungs

Strain (dose)	% of isolates[a] positive on day:										
	0	5	10	15	20	25	30	35	40	45	50
BP338 (low)	67	61	27	40	21	50	86	0	67	ND[b]	ND
BP357 (low)	0	14	11	17	25	0	0	—[c]	—	ND	ND
BP348 (low)	0	0	—	—	—	50	—	—	—	ND	ND
BP357 (high)	0	0	43	11	35	0	0	—	—	—	—
BP348 (high)	0	0	25	9	80	36	91	100	—	—	—

[a] Agglutination tests were performed on at least three bacterial isolates from each mouse.
[b] ND, Not determined.
[c] —, No bacteria recovered.

mice, and in this study, failure to produce FHA did not appear to change the rate of bacterial growth in the lungs of infant mice. Kimura et al. (3) have shown that FHA is a protective antigen for adult mice, as determined by reduced lung colonization after immunization with FHA. Combining their results with ours suggests that FHA is a protective antigen probably because it reduces the bacterial load by inducing opsonizing antibodies and not because of a requirement for neutralization of hemagglutinating activity.

Pertussis toxin does not appear to be required for initial growth in the lungs, but it could be responsible for the lethal effect since the course of the infection is similar to that of the wild type when bacterial loads are compared but radically different when lethal outcomes are compared. Pertussis toxin has also been shown to be a protective antigen in mouse studies (5), and our results suggest that neutralizing antibodies to pertussis toxin would be important for protection from disease.

The adenylate cyclase toxin has not been evaluated as a protective antigen because purified material has not been available until recently. The results of our studies using the adenylate cyclase toxin mutant are very intriguing. At low challenge doses, this factor appeared to be essential for colonization of the respiratory tract. In vitro assays have demonstrated that this toxin is a potent inhibitor of immune cell function (10) and suggest that elaboration of this toxin poisons the immune response and permits the bacteria to grow unmolested in the respiratory tract. However, when high challenge doses were examined, a seemingly contradictory result was obtained. The adenylate cyclase mutant appeared to survive and persist for longer periods than the wild-type strain, although less bacterial replication was observed.

Recent studies have shown that *B. pertussis* is able to invade and survive inside human cell lines (2). We have suggested that this may serve as the reservoir of the disease, since in this state the bacteria would be sheltered from the immune defenses. We also observed that mutants deficient in adenylate cyclase production actually invaded more successfully than did the wild-type strain (2). The apparent discrepancy between the results obtained with a low-dose challenge and those obtained with a high-dose challenge of the adenylate cyclase mutant can be explained as follows. Invasion of host cells may be a rather inefficient process, and at low challenge doses the immune response is sufficient to clear all of the bacteria from the lungs. At higher challenge doses, the immune response cannot clear all of the bacteria and the bacteria manage to invade the host cells. The bacteria in this protected environment are able to survive for very prolonged periods.

These studies have important vaccine implications. Con-

sistent with the results of the Swedish vaccine trials (1), failure to elaborate pertussis toxin, because of either mutagenesis (this study) or the presence of a neutralizing immune response (the vaccine trials), does not prevent infection by the bacteria but may result in a less serious course of disease. The untested implication of our results with the adenylate cyclase mutant is that a neutralizing response to this toxin could prevent both infection and disease. The availability of purified adenylate cyclase toxin should allow us to address this issue in future studies.

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